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# Pesticin Displays Muramidase Activity

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**Pesticin of *Yersinia pestis* is the only bacteriocin that converts sensitive cells to stable spheroplasts. The amino acid sequence of pesticin as derived from the nucleotide sequence shows no similarity to those of any of the bacteriocins. The unique properties of pesticin prompted an investigation of its mode of action. Since the pesticin plasmid does not encode a lysis protein for release of pesticin into the culture medium, pesticin was isolated from cells and purified to electrophoretic homogeneity. Highly purified pesticin degraded murein and murein glycan strands lacking the peptide side chains to products that were similar to those obtained by lysozyme, as revealed by high-resolution high-pressure liquid chromatography. After reduction of the murein degradation products with tritium-labeled sodium borohydride, acid hydrolysis, and separation of the products by thin-layer chromatography, radiolabeled muraminitol was identified. This indicates that pesticin is a muramidase, and not an *N*-acetyl-glucosaminidase, that converts cells into stable spheroplasts by slowly degrading murein.**

Pesticin of *Yersinia pestis* is determined by the pesticin activity gene (*pst*) and the immunity gene (*pim*) (22). The genes are tandemly arranged on small plasmids and transcribed in opposite orientation, which is typical for pore-forming colicins, as opposed to the colicins with nuclease activity (3, 16, 23). The pesticin determinant encodes an incomplete lysis gene; this explains the low amounts of pesticin released into the culture medium. The flanking regions of the pesticin genes show high sequence similarities to regions adjacent to the genes that determine the pore-forming colicins 10, 5, K, and E1 (19–22), indicating a common origin of these plasmids and exchange of colicin genes between these plasmids through recombination within the homologous regions. In contrast, the *pst* and *pim* genes themselves show no homology to any of the colicins, suggesting a unique activity of pesticin. Indeed, pesticin converts sensitive cells into osmotically stable spheroplasts (9). Pesticin shares this unusual bacteriocin activity with colicin M, which, however, rapidly lyses cells without osmotic protection (2). Unlike colicin M, which inhibits murein and O-antigen biosynthesis (10, 11), pesticin degrades murein (4). In accordance with the target of both bacteriocins in the periplasm, the immunity proteins of pesticin (22) and colicin M (8) are found in the periplasm, where they inactivate the incoming toxins before they reach their target. Since pesticin displays a unique activity among the bacteriocins, we analyzed its mode of action. Previously, it was shown that pesticin is a glucosaminidase that degrades murein (4). We confirmed cleavage of the murein polysaccharide strands, but our data are consistent only with a muramidase activity.

## MATERIALS AND METHODS

**Purification of pesticin.** *Escherichia coli* BL21 (27) was transformed with plasmid pUH64, which encodes the pesticin genes *pst* and *pim* downstream of the gene 10 promoter of phage T7. Since the *E. coli* strain does not express the pesticin receptor, it is resistant to pesticin even when more pesticin is produced than immunity protein. Cells were grown at 37°C in TY medium (1% Bactotryptone, 0.5% yeast extract, 0.5% NaCl [pH 7]) to an optical density at 578 of 0.5. Synthesis of phage T7 RNA polymerase, encoded on the BL21 chromosome under *lac* control, was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 2 h of growth, cells were harvested by centrifugation and suspended in 50 mM sodium phosphate buffer (pH 8.2) (SP buffer). Pesticin was isolated from cells since only about 5% of the pesticin activity was in the culture medium and the remainder was cell bound. Cells were disrupted by ultrasound treatment (three times for 20 s on ice). Cell debris was removed by centrifugation at 10,000 × *g* for 10 min, and the proteins in the supernatant were precipitated with 50% ammonium sulfate. The sediment was dissolved in SP buffer and desalted on a HiTrap column (Pharmacia, Uppsala, Sweden). The desalted solution was chromatographed on a ResourceQ anion-exchange column (Pharmacia) with SP buffer. The peak fractions containing the highest protein concentrations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with Coomassie blue (17). Only one protein band was visible. Purification of pesticin was examined by spotting serial dilutions of the samples on TY agar plates seeded with *E. coli* 5K transformed with plasmid pHM10, which encodes the pesticin receptor FyuA, rendering *E. coli* K-12 pesticin sensitive (24).

Chromatography of the peak fractions on a ResourceS cation-exchange column in 50 mM sodium acetate (pH 4.5) yielded a single peak. As a control, *E. coli* BL21 cells transformed with the vector plasmid Bluescript SK+ were grown, disrupted, treated with ammonium sulfate, and chromatographed in the same manner described for *E. coli* BL21(pUH64).

**General murein hydrolase assay.** [$^3$H]$A_2$pm-labeled murein sacculi (5 μg; 10,000 cpm) were incubated in the presence of enzyme samples (5 to 10 μl) in 100 μl (total volume) of 10 mM sodium acetate buffer (pH 4.7) for 30 min at 37°C. To precipitate unreacted substrate, 100 μl of a cetyltrimethyl ammonium bromide (CTAB) solution (1%) was added and the samples were kept on ice for 20 min (18). After centrifugation in an Eppendorf centrifuge for 20 min at 6°C, 100 μl of the supernatant was added to 1.5 ml of scintillation cocktail, and the radioactivity was determined in a TriCarb (Beckman) scintillation counter.

**Enzymatic hydrolysis of murein sacculi.** Murein sacculi (2 mg), prepared as described previously (7), were incubated in the presence of pesticin (0.4 mg/ml; purified by anion-exchange chromatography) in 1.3 ml (total volume) of 10 mM sodium acetate buffer (pH 4.5) for 16 h at 37°C. As a control, an analogous sample obtained from a preparation of *E. coli* cells that did not synthesize pesticin (see above) was incubated in the same way. Murein was digested with lysozyme by incubating 2 mg of murein sacculi in the presence of 1 mg of hen egg white lysozyme in 1.35 ml of 70 mM sodium phosphate buffer (pH 7.2) for 16 h at 37°C. For an incomplete digestion, 5 mg of murein was incubated in the presence of 20 μg of lysozyme in 1 ml of 50 mM sodium phosphate buffer (pH 6.2) for 14 h at 37°C. Samples were centrifuged at 300,000 × *g* for 30 min at 20°C, and the supernatant was boiled for 10 min, cleared by centrifugation in an Eppendorf centrifuge, and concentrated to 1 ml in a SpeedVac apparatus.

**Enzymatic hydrolysis of poly-(GlcNAc-β1,4-MurNAc) glycan strands.** Radiolabeled murein glycan strands were prepared from sacculi, tritium labeled in the amino sugars, by hydrolysis with human serum amidase as described previously (12). The glycan strands were fractionated by reverse-phase high-pressure liquid chromatography (HPLC) by the method of Harz et al. (12) and desalted by gel filtration chromatography on a P2 column (BioRad, Munich, Germany). A frac-

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tion of glycan strands with a length of 11 disaccharide units, (GlcNAc-β1,4-MurNAc)$_{10}$-β1,4-GlcNAc-β1,4-1,6-anhydro-MurNAc, was used as a substrate for pesticin. An appropriate amount of the glycan strand fraction was incubated in 105 μl of 20 mM sodium acetate buffer (pH 4.5) with 10 μg of purified pesticin for 5 h at 37°C. A second digestion was done with 2.5 μg of pesticin for 20 min. Hydrolysis of the glycan strands with lysozyme (0.4 μg/ml) was performed in 50 μl of 50 mM sodium phosphate buffer (pH 6.5) for 20 min at 37°C. After incubation, the samples were boiled for 5 min and cleared by centrifugation in an Eppendorf centrifuge for 5 min, and the supernatant was reduced with sodium borohydride ($NaBH_4$) (see below).

**HPLC fractionation of muropeptides.** Muropeptides were diluted 1:1 with sodium borate buffer (pH 9.0) and reduced with sodium borohydride (1 to 2 mg) for 30 min at room temperature. To stop the reaction, the pH was adjusted to between 3 and 4 with 20% phosphoric acid. Samples were made up to 150 μl, and aliquots (140 μl) were injected for HPLC fractionation on Hypersil ODS (3 μm). Muropeptides were separated as described by Glauner (6).

**HPLC fractionation of saccharides.** Reduced saccharides were separated on a Nucleosil (5 μm) column (25 by 0.46 cm) as described by Harz et al. (12) and monitored with a flowthrough scintillation counter (Flo One Beta, Canberra, Australia).

**Reduction and labeling of muropeptides with NaB[$^3$H]$_4$.** Samples were hydrolyzed with 4 N HCl at 104°C for 14 h in sealed glass ampules. Acid was removed by repeated drying in a SpeedVac and dissolving in 150 μl of $H_2O$; samples were finally dissolved in 100 μl of $H_2O$. After the addition of 5 μg each of unlabeled glucosaminitol and muraminitol, aliquots were applied onto silica 60 thin-layer plates (0.25 mm thick, 20 by 20 cm; Merck, Darmstadt, Germany). Chromatography was performed in butanol-pyridine-water-acetic acid (60:45:30:4; by volume). When the solvent front reached 18 cm, the plates were removed, dried at 60°C, briefly soaked in 0.5% ninhydrin solution (98% acetone, 2% acetic acid), and developed for 2 h at 80°C. Spots (3 by 3 mm) corresponding to the positions of glucosaminitol and muraminitol were cut out, and the radioactivity was determined after the addition of 1.5 ml of scintillation cocktail as described above.

## RESULTS

**Murein hydrolase activity of pesticin.** The pesticin sample used for the determination of the mode of action was highly purified and contained only a single protein band, as revealed by SDS-PAGE (Fig. 1). Murein hydrolases that cleave glycosidic or interpeptide bonds in murein degrade high-molecular-weight murein sacculi to small muropeptides. In the case of radioactively labeled sacculi, the increase in radioactivity in the supernatant after precipitation with CTAB can be taken as a measure of murein hydrolase activity (18). When isolated pesticin was added to [$^3$H]$A_2$pm-labeled murein sacculi, 50.5% of the radioactivity was released after incubation for 30 min at 37°C under the conditions described in Materials and Methods. By contrast, less than 1% of the sacculi were solubilized when a sample of the control preparation was incubated.

**Pattern of muropeptides released from murein sacculi.** When isolated murein sacculi were digested with pesticin (Fig. 2A) or hen egg white lysozyme, the patterns of released muropeptides obtained by HPLC separation were quite similar to each other, in particular when the pesticin digest is compared with an incomplete lysozyme digestion (Fig. 2C). In the area where monomeric and dimeric muropeptides were eluted (peaks 1 to 5), all peaks were present in both the lysozyme and the pesticin sample in similar ratios. The unresolved material that eluted after 70 min, representing mostly oligosaccharide muropeptides, is indicative of an incomplete digestion of the murein sacculi. As a control, samples obtained by the same isolation procedure as those used for the pesticin digestion, but from a culture harboring only the vector, did not produce significant amounts of muropeptides (Fig. 2B). This indicates that the observed muropeptide profile is due to the isolated pesticin and not to any contaminating endogenous murein hydrolases.

**Degradation of murein glycan strands.** Lysozyme and some of the murein hydrolases present in *E. coli* hydrolyze isolated murein glycan strands lacking the peptide moieties as well as murein sacculi (13, 25). Mostly di-, tetra-, and hexasaccharides accumulate by the action of the endomuramidase lysozyme,

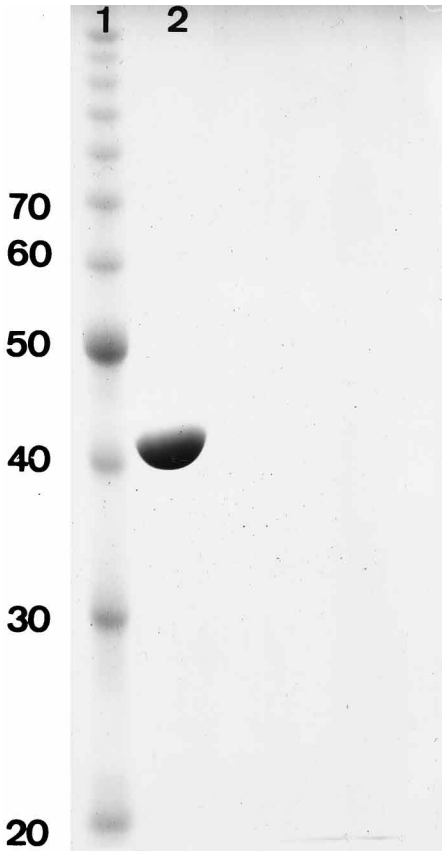


FIG. 1. SDS-PAGE of purified pesticin after column chromatography on ResourceQ (lane 2). The electrophoretic position of a 10-kDa protein ladder (Gibco-BRL, Eggenstein, Germany) is shown in lane 1.

with tetrasaccharide being the major product (Fig. 2C). By contrast, through the action of exomuramidases, disaccharides accumulate from the very beginning of the reaction (1, 25). As shown in Fig. 3, pesticin released a pattern of products (Fig. 3B) that was comparable to the pattern produced by lysozyme (Fig. 3C). The formation of di-, tetra-, hexa-, and some oligosaccharides indicates an endoglycosylase type of enzyme. The pesticin digest contained products from the 1,6-anhydromuramic acid ends of the glycan strands that eluted with elution times identical to those of the 1,6-anhydrodi-, 1,6-anhydrotetra-, and 1.6-anhydrohexasaccharide, which can be released only by a muramidase. By contrast, a glucosaminidase would release 1,6-anhydrosaccharides with an uneven number of sugar units.

**Enzymatic specificity of pesticin.** Enzymatic hydrolysis of both murein sacculi and murein glycan strands by pesticin released products that were qualitatively as well as quantitatively similar to the reaction products produced by the action of lysozyme. These results strongly suggested that pesticin has lysozyme activity. To unequivocally determine the enzymatic specificity of pesticin, the reaction products were reduced with tritium-labeled sodium borohydride (NaB[$^3$H]$_4$). Since the reducing ends of the glycan strands in the substrate are blocked by 1,6-anhydromuramic acid (7, 14), reducing ends can only be formed by the action of glycosylases, and reducing ends differ depending on the specificity of the enzyme. A muramidase releases products with a free hydroxyl group at carbon $C_1$ of the muramic acid, while a glucosaminidase releases products with a free $C_1$ hydroxyl group at a glucosamine. Hence, labeled muraminitol will be formed in the case of muramidase reaction

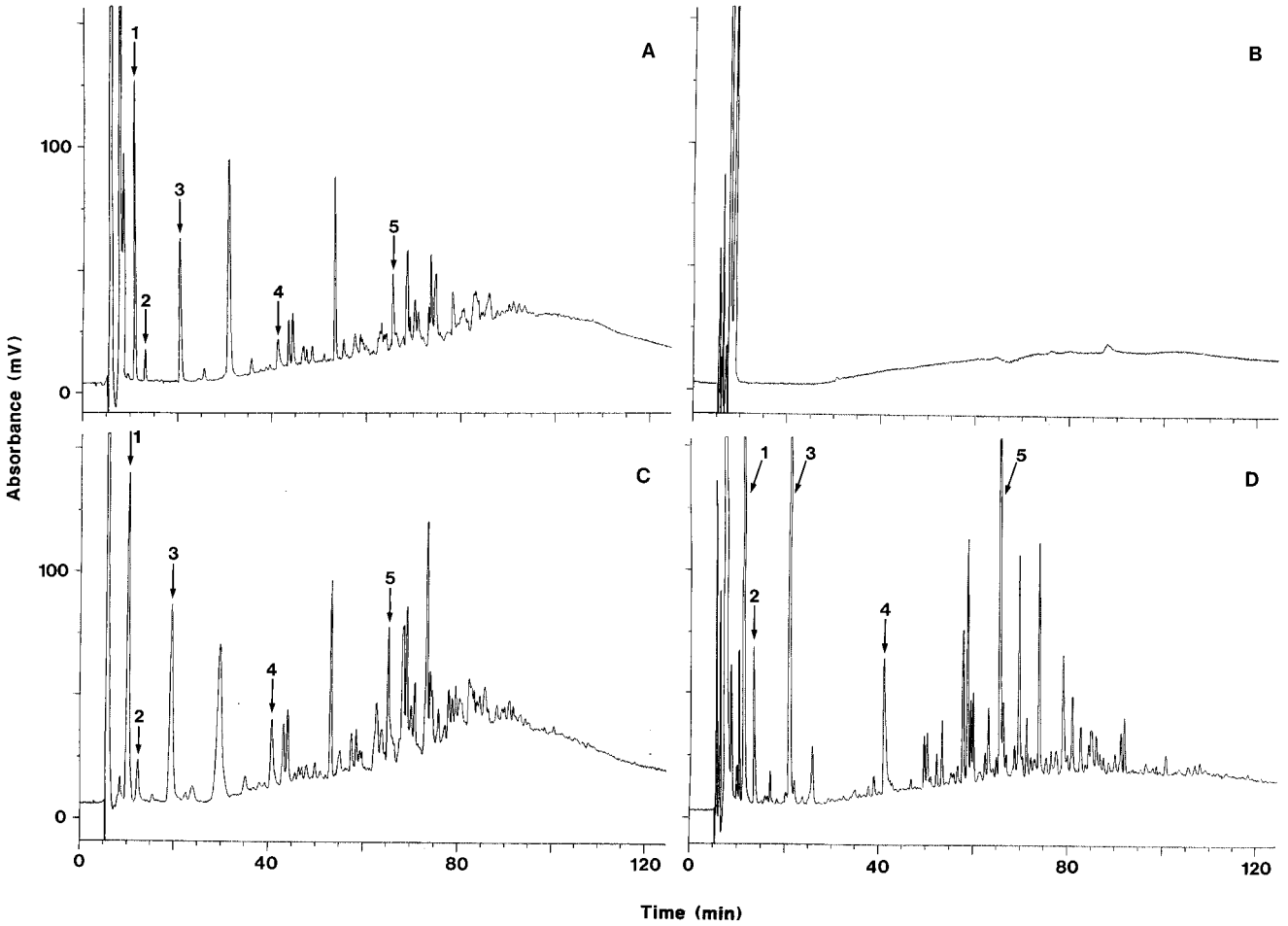


FIG. 2. Analysis of the reaction products released by hydrolysis of murein sacculi with pesticin. Murein sacculi were incubated with either pesticin (A), a control preparation (B), or lysozyme, both partial digestion (C) and a complete digestion (D) being shown. The reaction products were reduced with sodium borohydride and fractionated by reverse-phase HPLC on Hypersil ODS (3 μm) as described in Materials and Methods. Numbers indicate defined muropeptides: 1, GlcNAc-(β1,4)-MurNAc-L-Ala-D-Glu-m-$A_2$pm; 2, GlcNAc-(β1,4)-MurNAc-L-Ala-D-Glu-m-$A_2$pm-Gly; 3, GlcNAc-(β1,4)-MurNAc-L-Ala-D-Glu-m-$A_2$pm-D-Ala; 4, GlcNAc-(β1,4)-MurNAc-L-Ala-D-Glu-m-$A_2$pm-L-Lys-L-Arg; and 5, dimer of muropeptide 1 cross-linked by a D-Ala-m-$A_2$pm peptide bridge.

products, and labeled glucosaminitol will be formed in the case of glucosaminidase products.

After acid hydrolysis of the NaB[$^3$H]$_4$-reduced reaction products, analysis of amino sugars by thin-layer chromatography clearly revealed that pesticin releases products with the muramic acid at the reducing end (Table 1), demonstrating that pesticin has a lysozyme activity.

## DISCUSSION

The pattern of murein and murein glycan degradation products obtained with pesticin was similar to the pattern obtained with lysozyme and differed from the pattern expected from degradation by a glucosaminidase. The assignment of the degradation products by HPLC to certain murein structures is based on a large body of experience gained with a variety of murein hydrolases (12). The results of the borohydride reduction, which without any doubt indicated muraminitol as the product, were in agreement with the identification of the degradation products. This product could be obtained only after glycan cleavage at the $C_1$ position of *N*-acetyl-muramic acid. The former conclusion that pesticin has an *N*-acetylglucosaminidase activity rested on less-advanced methods, except for borohydride reduction, after which no glucosamine was found in hydrolyzed samples (4). It is less likely that a contaminating *N*-acetylglucosaminidase caused these aberrant results. The sample used was prepared by a procedure that resulted in two distinct chromatographic fractions that were interconvertible and in a third fraction; the fractions were very similar with regard to their amino acid compositions, two-dimensional peptide maps, antigenicities, and molecular masses (44 and 45 kDa on SDS gels [15], which is close to that we obtained [42 kDa] and the calculated value [40 kDa] from the derived amino acid sequence [22]). Pesticin hydrolyzed isolated murein slowly. A low reaction rate may explain why pesticin caused formation of osmotically stable spheroplasts of *E. coli* ϕ, *Yersinia pseudotuberculosis*, *Y. enterocolitica*, and *Y. pestis*. Cells are killed after 2 h of pesticin treatment, as revealed by plating pesticin-treated cells on nutrient agar plates (9). Colicin M kills cells more rapidly (after a 20-min incubation), and spheroplasts have to be protected from lysis by 15% sucrose (2). The turbid lysis zones caused by pesticin-synthesizing cells on a lawn of sensitive bacteria come from the small fraction of released pesticin and the low level of activity. It is less likely that the uptake rate into sensitive cells plays a role, since naturally sensitive *E. coli* strains as well as *Yersinia* strains show similarly low levels of sensitivity.

Colicins of the same activity type (pore formers or nucle-

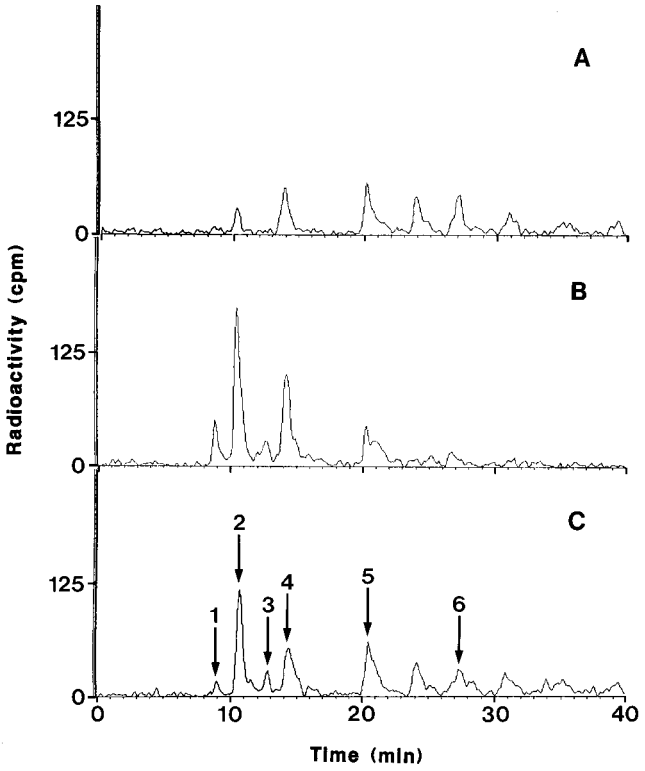


FIG. 3. Hydrolysis of murein glycan strands by pesticin. Murein glycan strands with a length of 11 disaccharide units, (GlcNAc-β1,4-MurNAc)$_{10}$-β1,4-GlcNAc-β1,4-1,6-anhydro-MurNAc, were incubated at 37°C with two different amounts of pesticin (A, 2.5 μg for 20 min; B, 10 μg for 5 h) or lysozyme (0.02 μg for 20 min). The samples were reduced with sodium borohydride and fractionated by reverse-phase HPLC on Nucleosil (5 μm) as described in Materials and Methods. Numbers refer to defined saccharides. 1, GlcNAc-β1,4-MurNAc; 2, (GlcNAc-β1,4-MurNAc)$_2$; 3, GlcNAc-β1,4-1,6-anhydro-MurNAc; 4, (GlcNAc-β1,4-MurNAc)$_3$; 5, GlcNAc-β1,4-MurNAc-β1,4- GlcNAc-β1,4-1,6-anhydro-MurNAc; and 6, (GlcNAc-β1,4-MurNAc)$_2$ GlcNAc-β1,4-1,6-anhydro-MurNAc.

ases) display high sequence similarities in domains responsible for colicin uptake, activity, and immunity. Between two colicins, one domain can be identical, while the remainder of the molecules are rather different; this led to the proposal that colicins were assembled from DNA fragments that encode functional domains (19, 21, 26). Considering this aspect, it is interesting to note that the entire sequence of pesticin does not resemble that of any colicin. Only the pentapeptide representing the TonB box is identical to the TonB box of colicin B (22); this and the resistance of *tonB* and *exbB* mutants to pesticin (5) assign pesticin to the group B colicins. Apparently, pesticin evolved without exchange of DNA fragments with colicins, although the *pst*- and *pim*-encoding plasmid pPCP1 is very similar in size and structure to the plasmids pColE1, pCol5, pCol10, and pColK (22).

TABLE 1. Analysis of the reduced murein degradation products by thin-layer chromatography[a]

| Enzyme | Radioactivity in NaB[$^3$H]$_4$-reduced amino sugars (cpm) | |
|---|---|---|
| | Glucosaminitol | Muraminitol |
| Pesticin | 584 | 2,633 |
| Lysozyme | 247 | 1,976 |
| Control | 308 | 464 |

[a] Murein sacculi were incubated with lysozyme or pesticin and reduced with NaB[$^3$H]$_4$. The enzymatic digests were hydrolyzed with 4 N HCl for 14 h at 104°C and separated by thin-layer chromatography on silica plates as described in Materials and Methods. The spots corresponding to muraminitol ($R_f$ = 0.235) and glucosaminitol ($R_f$ = 0.101) were excised, and the radioactivity of the material was determined. The control consisted of an *E. coli* BL21 extract that does not contain pesticin.

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